REDUCED STATURE PLANTS

The present invention relates to the control of plant size through the gibberellin-biosynthetic pathway.

Although large trees can produce large quantities of fruit or nuts, for example, such things as harvesting the crop and effectively targeting insecticidal sprays, can be difficult. By contrast, smaller trees are not only easier to harvest from and to spray, but can often be more prolific, as well as produce fruit of higher quality, and are easier to manage than the traditional large trees.

Thus, it is generally desirable to produce smaller trees, and this can be achieved by the use of dwarfing rootstocks, in combination with pruning and training. Nevertheless, this is generally insufficient, and it has still proven necessary to use chemical growth retardants to provide extra control of shoot growth on many fruit tree varieties.

Growth retardants are generally critical for the control of vigour in fruit trees, but there is a general desire to avoid the use of chemicals, wherever possible. Further, the commonly used retardant, daminozide, was voluntarily withdrawn by the manufacturer in 1989 from use as a fruit tree retardant, owing to concerns over its possible toxicity. The use of paclobutrazol has also been restricted, owing to its extreme persistence in the soil. Other growth retardants, such as Regalis (prohexadione calcium), are currently in trial, but await approval.

It would also be desirable to be able to use rootstocks which were either tailored to the local conditions, such as providing drought or pest resistance, or which were particularly vigorous. The need to use dwarfing rootstocks generally outweighs any possibility of selecting rootstocks for other reasons.

Hedden, *et al.*, review gibberellin biosynthesis (Trends in Plant Science (2000), 5:12, 523-30), including the observation that inhibiting the 20-oxidase in the gibberellin pathway can lead to dwarfism in Arabidopsis.

WO 01/66777 discloses the over-expression of giberrellic acid (GA) 20-oxidase to increase growth in trees.

WO 03/006655 discloses that a mutation in the rice C-20 oxidase gene leads to semi-dwarfism.

EP 1254958 also discloses that mutants of GA 3-oxidase genes lead to semi-dwarfism in rice.

Surprisingly, it has now been found that it is possible to inhibit the production of gibberellin in the scion, without the rootstock being able to fully reverse the dwarf phenotype.

Thus, in a first aspect, the present invention provides a dwarf plant, comprising a rootstock and a scion grafted thereon, wherein gibberellin levels in the scion are reduced.

The plants of the invention are preferably trees, but generally be any woody plant where a scion is graftable, or grafted, onto a rootstock. While the plant created in this manner may consist of two varietals of a single species, or even one varietal wherein the scion exhibits reduced levels of gibberellin, but where the rootstock is not so restricted, it may also comprise a rootstock generally from within the same or a related genus, the only requirement being that the scion be capable of "taking" on the rootstock. Thus, provided that the scion can grow when grafted onto the rootstock, it is immaterial from which species either comes.

Any tree that produces a commercial harvest is suitable for use in the present invention, and examples include fruit trees, particularly of the *Citrus* and *Malus* genera, including oranges, lemons, limes, grapefruit, pomelos, apples, plums, peaches, almonds, and cherries, with dessert, culinary and cider cultivars of apples being particularly preferred.

It will be appreciated that reference to a "tree" or "trees" herein includes reference to other plants of the invention, unless otherwise apparent or specified.

While it is generally preferred that the rootstock for an apple also be an apple, it is also feasible to use a pear rootstock, for example, or *vice versa*.

It is a particular advantage of the present invention in that it is possible to select a non-dwarfing rootstock. This has not previously been possible, the primary attribute a dwarfing rootstock being just that. With the present invention, it is now possible to select a rootstock for any desired property, without also having to select for dwarfism.

The gibberellin levels are reduced in the scion *via* the gibberellin biosynthetic pathway. It is particularly preferred that the parent plant from which the scion is derived

be obtained by transformation to achieve the reduced levels of the selected gibberellin(s). As discussed below, this may either involve inhibiting GA synthetic enzymes, either by inhibiting their action or by inhibiting their production, or by enhancing the effect of enzymes having the selected GA as substrate, particularly by over-expression of that enzyme. It will be appreciated that any number of enzymes may be targeted in this manner, although it is generally sufficient to target only one. Thus, reduced levels may be effected either by inhibiting gibberellin production, or by encouraging enzymatic processing of the target gibberellin, or both.

In an alternative aspect, the present invention provides a dwarf tree, comprising a rootstock and a scion grafted thereon, wherein the production of gibberellin in the scion is inhibited.

It is particularly surprising that the rootstock does not restore full growth potential to an inhibited scion, given that it is the rootstock that is used to inhibit the growth of the scion, rather than selecting a dwarf tree from which to take a scion.

Indeed, it is preferable to select the cultivar, or varietal, for the scion from bountiful croppers and those otherwise known for the excellence of their harvest. Such cultivars are rarely associated with restricted growth, and any restriction on the growth potential of such cultivars would be expected to be overcome by a vigorous rootstock. Surprisingly, this assumption turns out not to be correct, and substantial dwarfism induced by inhibition of gibberellin production is not completely overcome by even the most vigorous of rootstocks. For example, a plant that only grew to about 25% of the height of the wild type may grow more on a vigorous rootstock, but maybe to about 50 – 70%, for instance.

It is particularly advantageous that the invention permits the utilisation of any rootstock for the cultivation, for example, of commercially viable, perennial fruit or nut crops as scions. By controlling the stature of the scions independently of the rootstock it makes it possible to select a rootstock for purposes other than to dwarf the resulting tree. This assists in reducing the requirement for insecticides, and allows the rootstock to be selected for suitability to the local terrain, or any other desirable trait, which may be something as simple as vigour, but may alternatively, or in addition, include such properties as drought resistance, pest and disease resistance or toxin resistance, such as salinity resistance, for example, thereby reducing artificial inputs and pollution.

The availability of self-dwarfing fruit scion varieties permits a much greater range of rootstocks to be used with the scions. For example, invigorating rootstocks, such as M25, could be used which provide resistance to soil-borne pests and diseases and better anchorage than existing commercial rootstocks.

Inhibition of the gibberellin biosynthetic pathway can take place at any suitable point, but it is generally preferred to restrict the interference to the latter stages of the biosynthetic pathway, in order to obtain moderate, rather than extreme, reductions in growth. Indeed, the GA pathway is complex, and it is preferred to limit interference to the 20-oxidase, 2-oxidase and 3-oxidase, but it is particularly preferred to inhibit the 20-oxidase. GA 2-oxidase inactivates GA's, so that a scion in which GA 2-oxidase is over-expressed will exhibit reduced levels of GA, in accordance with the present invention.

By inhibition of the production of GA is meant that at least one form of GA necessary for growth of the scion is produced in a lesser quantity than in the corresponding wild type. This may take the form of complete blocking of synthesis of that GA, or a reduced production, such as between 10 and 50%, for example. This reduction may also be achieved by suitable expression, or over-expression, of a degradative enzyme in the scion.

Although any GA may be targeted in the present invention, provided that a reduced amount of that GA results in reduced growth of the tree, the preferred GA's to target are GA_1 and GA_3. It is also generally preferred that the effect on the tree is to reduce the internodal distance and, preferably, the amount of nodes, as well.

Inhibition of the production of the GA is preferably effected by interfering with an enzyme in the GA pathway, such as one of those identified in the reaction scheme, below.

The enzyme may be inhibited by any suitable means. In plants, this is generally restricted to transformation, especially using *Agrobacterium tumefaciens*, and methods for using this organism are well documented. Transformation of cropping trees, such as fruit or nut trees, is readily achieved by taking fresh samples of healthy, growing leaves, incubating with a suitably transformed preparation of *A. tumefaciens*, then cultivating the transformed tissue. Incorporating a suitable marker, such as kanamycin resistance, enables positive transformants to be selected.

The transformation may take the form of incorporating a targeted mutation or a gene encoding an inhibitor for the selected enzyme, for example. In a preferred embodiment, the tissue is transformed with an antisense nucleotide sequence such as to generate an antisense RNA for the mRNA encoded by the naturally occurring gene. The resulting dsRNA is automatically digested by the cell in what is assumed to be a legacy of protection against viruses, thereby preventing, or substantially inhibiting, the production of the necessary enzyme, and thereby inhibiting the production of the GA.

The antisense DNA is suitable preceded by an appropriate promoter and may be accompanied by a termination sequence.

The size of the antisense sequence is not important to the present invention, provided that the sequence is capable of binding the target mRNA, *in vivo*, in a manner sufficient to trigger the dsRNA digestion response. The amount of nucleotides required to achieve a dsRNA response varies according to the technique used, and will be apparent to those skilled in the art. A typical length for an interfering RNA (RNAi) may be between about 15 and several hundred nucleotides, but short lengths of about 25 are known to be effective as RNAi's in effecting suppression. A recognition motif in a ribozyme may only require about 10 – 15 nucleotides, for example. About 50 – 100, or greater, nucleotides are generally required for site specific recognition, and it is preferred that the sequence be substantially faithful to the target, in order to ensure the dsRNA effect, although some degeneracy is permissible, provided that the gene is at least partially silenced. More particularly, it is often preferable to generate a hairpin construct which, as an example, might comprise about 50bp to 100bp, so that the DNA would provide a promoter, followed by 50 to 100bp sequence-X-50 to 100bp sequence X, the second 50 to 100bp sequence being inverted, followed by a terminator.

Such techniques are referred to herein as gene silencing, and may also be achieved by transforming the scion tissue with a gene encoding a suitable *trans*-acting ribozyme targeted against the mRNA of the GA 20-oxidase gene, for example.

Overexpression of a full-length sense transgenic copy of an endogenous gene can result in an increase in abundance of the target mRNA. However, an alternative outcome is the triggering of post-transcriptional gene silencing (PTGS), involving small antisense RNAs, which reduce RNA accumulation by promoting the destruction of both transgenic

and endogenous sense RNA (co-suppression). Although the mechanism of this is not completely clear, it is likely that PTGS is triggered by the production of some antisense RNA by the transgene, and it appears likely that this is what is happening in the sense constructs in the accompanying Examples. In the case of the apple sense construct in the Examples, the endogenous gene is not being duplicated but, rather, an internal fragment of Ms20ox1 is being expressed which appears to efficiently trigger PTGS (*c.f.* Han Y.H. and Grierson D. (2002) Relationship between small antisense RNAs and aberrant RNAs associated with sense transgene mediated gene silencing in tomato. Plant J., *29*, 509-519). Thus, the present invention also contemplates silencing by co-expression, using a partial or full length sequence.

Other forms of inhibition may be used, as appropriate, and may include the expression of suitable antibodies, for example, such as single chain antibodies directed against a target GA to sequester, and thereby inactivate, that GA.

As noted below, there are various isozymes located throughout even an individual plant, and it is preferred to target those isozymes primarily found in shoot tips. Isozymes located in anthers but not in shoot tips, for example, are unlikely to have any significant effect on the overall size of the cultivar. It will be appreciated that individual enzymes or families of enzymes may be targeted by a single RNAi, for example, by suitable selection of a consensus sequence.

The transformation may also be with a gene encoding an inactive version of the enzyme, but which has a greater affinity with the substrate, for example.

Any promoter capable of promoting expression in the appropriate scion may be used in preferred embodiments of the present invention. Where constitutive promoters are desired, then a suitable example is the 35S promoter, from the cauliflower mosaic virus. However, where it is desired to reduce expression of an enzyme that can have severe stunting effects on a plant, then it may be desirable to use a tissue specific promoter. It will be appreciated that a tissue specific promoter need not be completely specific to a given tissue, rather that it be proportionately more active in the selected tissue than other tissues. Preferred tissue specific promoters have substantially higher activity in their target tissues, and are preferably substantially inactive in other tissues.

Examples of tissue specific promoters are provided, for example, by Gittins, J. R., *et al.* (Transgene expression driven by heterologous ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene promoters in the vegetative tissues of apple, Planta (2000) Volume 210, pp 232-240), and include the heterologous ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit (SSU) gene promoters, RBCS3CP (0.8-kbp) from tomato and SRS1P (1.5-kbp) from soybean. These heterologous SSU promoters were found to be active primarily in the green vegetative tissues of apple, although activity in the roots was noticeably higher with the RBCS3C promoter than the SRS1 promoter. The activity of the SRS1 promoter appears to be dependent on light, unlike the RBCS3C promoter.

It is generally preferred that the expression product effecting the gene silencing is associated with a tissue specific promoter, such as an SSU promoter.

We used a transgenic approach to reduce the levels of bioactive GA's in the scion variety, resulting in significant reductions in stem height. After grafting such dwarfed scions onto normally invigorating rootstocks, we have shown that the scion is still dwarfed, with control plants of the same cultivar displaying the expected vigour when grafted onto these rootstocks. This, then, permits the use of any available rootstock or the growth of any perennial crop variety on their own roots to obtain dwarf trees without the need for the application of chemical growth retardant. A dwarfed scion is easier to manage and is generally earlier cropping.

In a preferred embodiment, dwarfing is achieved by suppression of GA 20-oxidase gene expression. In one embodiment, lower endogenous levels of bioactive GA's were produced in the growing areas of the plant. GA 20-oxidases catalyse three successive oxidative reactions in the conversion of GA_{12} to GA_9 *via* GA_{15} and GA_{24}, and of GA_{53} to GA_{20} *via* GA_{44}, and GA_{19}. GA_{17} and GA_{25} are formed as inactive side-products (reaction scheme, *infra*). GA_9 and GA_{20} are then converted by GA 3-oxidases (3ß-hydroxylation) to bioactive GA.

Levels of native bio-active GA were reduced in Greensleeves apple (dessert variety) by co-suppression and anti-sense strategies with a 314bp long DNA fragment of an endogenous GA 20-oxidase gene sequence (referred to as '20ox1'). This caused dwarfism in some of the resulting transgenic plants where silencing of endogenous GA

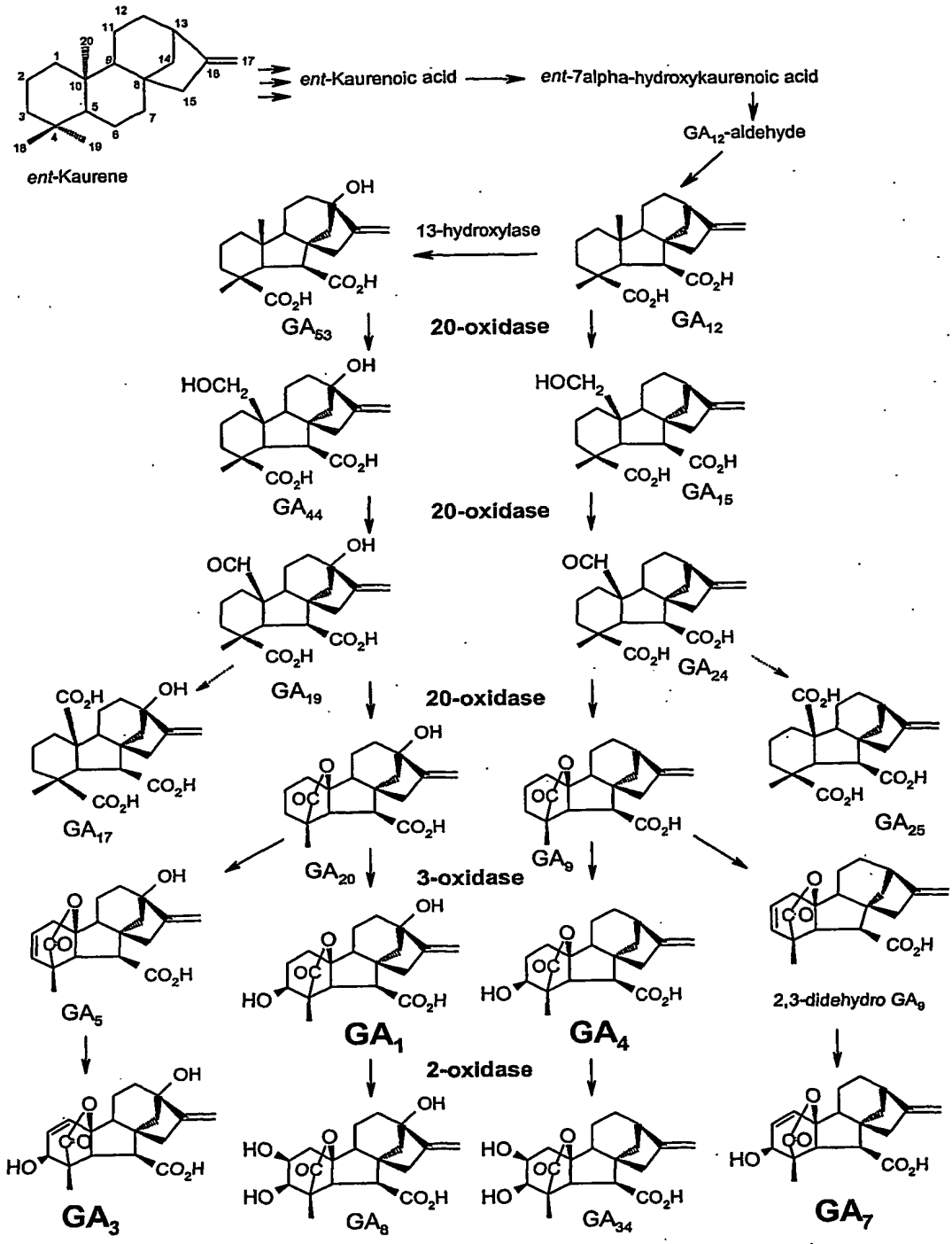
20-oxidase gene expression had occurred. It is generally important to silence the correct GA 20-oxidase gene because, in all plant species investigated to date, these genes exist as multi-gene families with different spatial, temporal and developmental patterns of expression. For example, two GA 20-oxidase gene cDNA sequences were isolated from Greensleeves that were shown to have quite different spatial patterns of expression. *MdGA20ox1* (corresponding to 20ox1) is expressed in developing embryos, shoot tips, and young leaves, with very low expression in ovary tissue just after pollination. In contrast, *MdGA20ox2* is expressed in stamens and unpollinated late balloon stage ovaries. Therefore, it is unlikely that silencing *MdGA20ox2* would cause dwarfism, as expression was not detected in shoot tips or young leaves.

It will be appreciated that the present invention also provides a process for obtaining a dwarf plant, wherein the plant is as defined and comprises a rootstock and a scion grafted thereto, the term "dwarf" indicating that the plant is of reduced stature by comparison with non-dwarfed plants of the same cultivar from which the scion is derived, comprising transforming the cultivar to exhibit reduced levels of gibberellin. The process will generally comprise transforming cultivar tissue, typically meristem, or stem, cells and culturing the tissue to obtain a plant, or plants. The invention extends to such transformed tissue, whether it be the cells, callus or a plant capable of providing a scion.

Methods for reducing the levels of GA are as exemplified above and elsewhere herein, and preferably comprise inhibiting or blocking expression of GA 20-oxidase, such as by PTGS, especially by RNAi.

The invention further provides plants produced by this process, as well as crops obtained from plants of the invention, and stands of such plants.

The following reaction sequence shows the late stages of the GA biosynthetic pathway involved in the production of bioactive $GA_{1,4,3,7}$. Both the non 13-hydroxylation pathway and the 13-hydroxylation pathway can occur in any particular species, but one or the other may predominate. GA_{17} and GA_{25} occur as minor by-products and have no known biological activity. The GA 20-oxidase, 3-oxidase and 2-oxidase genes exist as multiple gene families in the various species analysed so far. These dioxygenase enzymes utilise 2-oxoglutarate as a co-substrate, require Fe^{2+}, and are stimulated by ascorbate.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ent-7alpha-hydroxykaurenoic acid
GA12-aldehyde
13-hydroxylase
GA53
GA12
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GA44
GA15
GA19
GA24
GA17
GA20
GA9
GA25
3-oxidase
GA5
GA1
GA4
2,3-didehydro GA9
2-oxidase
GA3
GA8
GA34
GA7

Brief Description of the Figures:

Figure 1 shows the pDH51 vector as used to fuse the 20ox1 fragment with the cauliflower mosaic virus 35S promoter;

Figure 2 shows the pSCV1.2 binary vector. The 35S-20ox1-expression cassette was blunt ligated into the Sma1 site;

Figure 3 shows pSCV1.2-20ox1 construct T-DNA showing the 20ox1 anti-sense (→) and sense (←) expression cassette inserted into the *Sma*1 site by blunt ligation;

Figure 4 shows dwarfism in transgenic plants where silencing of endogenous GA 20-oxidase gene expression had occurred;

Figure 5 show that the dwarf scions remained dwarfed after a growth season, even on invigorating M25 rootstock; and

Figure 6 compares the relative effects on mean overall height and mean internode length between rootstocks, expressed as a percentage of the tallest non-transgenic control means.

The present invention is further illustrated in the following non-limiting Examples.

EXAMPLE 1

Apple Transformation

Transformation of Greensleeves

The procedures were performed under sterile conditions in laminar flow cabinets and class II downward flow cabinets.

Shoots were rooted, and young healthy leaf tissue was used, no longer than three weeks after transfer to R37. Four days before leaf disks were cut, *Agrobacterium tumefaciens* strain EHA101 (containing the appropriate pSCV construct) was streaked from a frozen stock onto LB agar plates containing 25mg.l^{-1} neomycin, 50mg.l^{-1} chloramphenicol, 25mg.l^{-1} gentamycin, and incubated at 28°C. After two days growth, one colony was inoculated in 5ml YEP media (10g.l^{-1} yeast extract [Oxoid Ltd.], 10g.l^{-1}

peptone [Oxoid Ltd.], 5g.l^{-1} NaCl [Sigma-Aldrich], pH7.2) containing 25µg.ml^{-1} neomycin, 50µg.ml^{-1} chloramphenicol and 15µg.ml^{-1} gentamycin, and was incubated at 28°C with shaking at 200rpm for one day. A 1ml aliquot was then inoculated into 9ml fresh YEP liquid media containing antibiotics as before and incubated at 28°C with shaking overnight.

On the day of transformation, the percentage transmission at 420nm of a 100µl aliquot of overnight culture was read in a spectrophotometer and the *Agrobacteria* culture was centrifuged at 3500xg for 15 minutes. The supernatant was discarded and the pellet was immediately re-suspended in a volume of virulence induction media (2.2g.l^{-1} MS salts [Sigma-Aldrich], 10g.l^{-1} sucrose [Sigma-Aldrich], 1mM betaine hydrochloride [Fischer] 0.1mM acetosyringone [Sigma-Aldrich], pH5.2), calculated by multiplying the absorbance$_{420}$ reading by 200ml.

The bacterial solution was aliquoted in 10ml volumes into sterile 15ml Falcon tubes (Sarstedt) and incubated at 20°C with gentle shaking for 5 hours. During this time, leaf disks approximately 7mm in diameter were cut with a sterile number 2 size cork borer from young healthy expanding leaves and placed abaxial side down onto BNZ 5-1-1 regeneration medium (5mg.l^{-1} BAP [Sigma-Aldrich], 1mg.l^{-1} NAA [Sigma-Aldrich], 1mg.l^{-1} TDZ [Sigma-Aldrich], 4.4g.l^{-1} MS salts [Sigma-Aldrich], 40g.l^{-1} sorbitol [Sigma-Aldrich], pH5.2, and 2.5g.l^{-1} Phytagel [Sigma-Aldrich]), contained in Petri dishes for holding until required.

After *Agrobacterium* induction, 50 leaf disks were placed in each tube of the induced bacterial solution and incubated at room temperature with gentle shaking for 20 minutes. Excess liquid was removed from each disk by blotting on sterile filter paper (Whatman) and the disk was placed abaxial side down onto filter paper (wetted with induction media) overlaid on BNZ 5-1-1 regeneration medium in Petri dishes. The plates were sealed and the disks were co-cultivated for three days at 25°C in darkness. After co-cultivation, the *Agrobacteria* were removed by washing the leaf disks in 500mg.l^{-1} Augmentin and 200mg.l^{-1} cefotaxime solution (pH5.2) with gentle shaking for 5 hours at 20°C. The leaf disks were then blotted dry as before and transferred to BNZ 5-1-1 regeneration media containing kanamycin and cefotaxime at 100mg.l^{-1} and 200mg.l^{-1} respectively.

The disks were incubated at 28°C in darkness and transferred to fresh media every 4 weeks. After approximately one month, shoots generally began to form from callus. Once of sufficient size, shoots were transferred to A17 media containing 50mg.l^{-1} kanamycin (Sigma-Aldrich) and grown on in light (16 hour day, 24°C, 16°C at night). Each shoot was recorded as a separate line and as such was kept separate from other lines.

Construct assembly

Insert preparation

Between 1 to 2μg of plasmid DNA was digested with the appropriate restriction enzyme, according to the manufacturer's instructions. After digestion with restriction enzymes the fragment to be ligated was gel purified using the dialysis bag method or the Qiaex II Gel Purification Kit (Qiagen). If blunt end ligation was required, then nucleotide overhangs left by restriction enzymes were removed by T4 DNA polymerase (Promega), following the manufacturer's instructions. Blunted fragments were extracted twice in phenol:chloroform:isoamyl alcohol (125:124:1, pH8) and precipitated as before. A small aliquot was run on a gel against λ DNA standards (Invitrogen Life Technologies™) in order to determine concentration.

Vector preparation

Between 1 to 2μg of the desired plasmid vector was digested with the appropriate restriction enzyme(s) and gel purified as before. If a blunt ligation was required, the linearised vector was blunted as before. The linearised vector was de-phosphorylated with calf intestinal alkaline phosphatase (CIAP, Promega) for 30 minutes at 37°C according to the manufacturer's instructions. After de-phosphorylation of the free ends to prevent self re-ligation, the vector was phenol:chloroform extracted and re-suspended in water. To determine an approximate concentration, a small aliquot was run out on an agarose (Seakem) gel.

Ligation

Using T4 DNA Ligase (Promega) with 100ng of prepared vector, ligation reactions (20μl total volumes) were assembled with prepared insert at a 3:1 molar ratio to prepared vector. Additional ATP was included in each 20μl ligation reaction to a final reaction

concentration of 20μM and the reactions were incubated at 20°C overnight. The ligation reaction was transferred into *E. coli* (strain DH5α) *via* the '5 minute transformation protocol' (Pope and Kent 1996). The competent cells used were prepared as in Alexander *et al.* (1984).

Confirmation of ligation and transfer to Agrobacterium

Plasmid DNA from 12 resultant colonies for each orientation was prepared using the following STET method: Transformant colonies were streaked onto sectors of selective plates and incubated overnight at 37°C. A small amount of the streaked bacteria (approximately 20μl) was scraped using a sterile micropipette tip and placed in 100μl STET buffer (8% sucrose; 5% Triton X100; 50nM EDTA; 50mM Tris-HCl, pH8.0) in a 1.5ml Eppendorf tube and vortexed. The tip was discarded and 10μl of freshly prepared 10mg/ml lysozyme was added and the tubes were vortexed again and incubated at room temperature for 5 minutes. The samples were placed in boiling water for 1 min and then centrifuged at 14000xg for 15 minutes. The supernatant was transferred to a fresh tube and precipitated by adding an equal volume of propan-2-ol and mixed by vortexing. The samples were centrifuged as before and the pellets were washed in 70% ethanol (v/v). After brief drying under vacuum, the DNA pellet was re-suspended in 20μl TE buffer. Restriction analysis with *Eco*R1 identified the colonies containing the expression cassette in sense and anti-sense formation.

One colony for each orientation was grown overnight at 37°C in LB medium containing 50μg/ml ampicillin. Plasmid DNA was purified using a Plasmid Midiprep kit (Qiagen). Prepared constructs were transferred to the *Agrobacterium tumefaciens* strain EHA101 by electroporation in an E. coli Pulser™ Transformation Apparatus (Biorad) using 100 to 300ng of plasmid DNA according to the manufacturer's instructions. Competent *Agrobacterium* cells were prepared using the method described by McCormac *et al.* (1998).

Construct preparation: sense and anti-sense 20ox1 fragment, GA 2-oxidase

Two constructs (pSCV1.2-20ox1-sense/anti-sense) were made containing an apple GA 20-oxidase fragment, 314 base pairs long, in sense and anti-sense formation.

The sequence (SEQ ID NO. 1) was as follows:

TGCTCACAGTGGGGGCCAGTTCCTAAAGTCTGCTCAGGTTTCTGGCATGGTGG
GTAGTAATTAAGCCTCATTATCGAATCATTGTCTTCGAAAAACTCCTTGAAGTA
AGCTCTGTCGACTCCAAGGCTCAGTCCCAGAAGTTCCATGATCCCAATAGAAA
GTGTGCTCATAGCCTCACTATAATCTTGGTAAACCCTCCCGAATTCCTTGAATT
CTTCTCCCATTTTGTTGCAAAAATAATCTTGGATAATATTGATTGAGCCTTTTTC
GGCGGAGTAGCTGAAAGAGAGAGTCTCCTTCCACGGTAATTTGCA

This was derived from NEJA1 and NEJA3 degenerate primers and had been previously cloned into the vector pMOSBlue (Amersham). The fragment was excised by a *Sma*1/*Xba*1 double digest and blunt end ligated into to the *Sma*I site of pDH51, and Figure 1 shows this pDH51 vector as used to fuse the 20ox1 fragment with the cauliflower mosaic virus 35S promoter. Insertion into the multiple cloning site of pDH51 results in transcriptional fusion to the cauliflower mosaic virus 35S promoter and down stream from the multiple cloning site there is a CaMV polyA terminator sequence.

The respective expression cassettes (35S-20ox1-NOS) with the 20ox1 in sense and anti-sense were excised by *Nco*1/*Hind*III digest and blunt ligated into the *Sma*1 site of the pSCV1.2 transformation vector. Figure 2 shows the pSCV1.2 (plasmid Shell Clean Vector 1.2) binary vector (Gittins *et al.* 2000). The 35S-20ox1-expression cassette was blunt ligated into the Sma1 site. The appropriate final constructs were selected by restriction analysis with *Eco*RV and *Kpn*1, and later by PCR.

Finally, one of each orientation, as shown in Figure 3, was selected and transformed into the *Agrobacterium tumefaciens* strain EHA101 by electroporation. Figure 3 shows pSCV1.2-20ox1 construct T-DNA showing the 20ox1 anti-sense (→) and sense (←) expression cassette inserted into the *Sma*1 site by blunt ligation. The 20ox1 sequence contains two *Eco*R1 sites. The *Bgl*II sites were used later for DNA blot analysis of transgenic plants. LB= left border sequence, RB= right border sequence.

20-ox1 sense transformation

In one transformation of 300 Greensleeves leaf disks with the 20ox1 sense construct, a total of 69 separate lines regenerated. Of these, 15 were escapes and died under kanamycin selection when the shoots were transferred to A17 media containing 50mg.l^{-1} kanamycin. The rest were tested for the presence of *nptII* and 20ox1 fragments by PCR. Of the remaining 54 lines, 39 tested positive for both *nptII* and 20ox1 to give a

transformation frequency of 13% while 15 tested positive for *nptII* only. *nptII* only lines are the result of incomplete T-DNA transfer and are detected here because the *nptII* transgene (selectable marker) lies next to the right hand border, which is integrated first. Lines without the 20ox1 transgene were not analysed any further.

All the lines that grew under selection were transferred to the greenhouse to look for altered phenotypes and seven lines showed dwarfed phenotypes of varying severity (Table 1).

Table 1

Summary of dwarf lines produced by transformation with pSCV1.2-20ox1-sense

Line	*Dwarfing severity (lowest to highest, + to +++)*	*PCR for nptII*	*PCR for 20ox1*
A01	++	+	+
A10	++	+	+
D04	+	+	+
E05	+	+	+
H01	++	+	+
P01	+	+	+
Q01	+++	+	+

Confirmation of transgene insertion and estimates of copy number

DNA was extracted from young leaves collected from individual plants from dwarf lines and digested with *Bgl*II restriction enzyme. All the dwarf lines except D04 and E05 were confirmed to contain the 20ox1 transgene by *Southern* blot of *Bgl*II digested DNA and were estimated for copy number (Table 2). *Bgl*II was used because it cuts within the NOS terminator sequence and at the right hand border of the pSCV1.2-20ox1

transformation constructs (Figure 3). This allows an approximate estimate of copy number in transgenic plants to be made by counting the number of bands because cleavage at this point in the integrated construct and outside in genomic DNA results in different sized fragments depending where the transgene is inserted. However, this cannot distinguish cases where there are no *Bgl*II sites between two unlinked transgenes integrated in opposite orientations. The same is true for multiple insertions in tandem or inverted tandem repeats so these copy number estimates are approximate. Copy number was not investigated in any more detail.

Table 1

Estimated copy numbers of 20ox1-sense transgene transformed plants

Line	*Confirmation of 20ox1 transgene integration by DNA blot*	*Estimated copy number of 20ox1 transgene*
A01	yes	3
A10	yes	1
D04	not analysed	
E05	not analysed	
H01	yes	1 or 2
P01	yes	5-8
Q01	yes	1

It is possible that the 6kb band seen for line H01 and Q01 is due to a tandem inverted repeat. The length between the *Bgl*II site in the NOS terminator and the left border is approximately 3kb. A tandem inverted repeat would place the two 20ox1 transgenes in very close proximity, enough to allow dsRNA formation if read through transcription occurred.

EXAMPLE 2

The levels of native bio-active GA were reduced in Greensleeves apple (dessert variety) by co-suppression and anti-sense strategies with a 314bp long DNA fragment (SEQ ID NO. 1, *supra*) of an endogenous GA 20-oxidase gene sequence (referred to as '20ox1'). This caused dwarfism in some of the resulting transgenic plants where silencing of endogenous GA 20-oxidase gene expression had occurred (Figure 4). The GA 20-oxidase gene used was *MdGA20ox1* (corresponding to 20ox1) and is expressed in developing embryos, shoot tips, and young leaves, with very low expression in ovary tissue just after pollination.

The dwarfed plants grown on their own roots had reduced levels of bioactive GA_1 (Table 3).

Table 3

Concentrations ($ng.g^{-1}$ fresh weight) of GAs in 20ox1 sense transgenic dwarf lines H01 and P01, and anti-sense 20ox1 transgenic AS13 plants compared to their respective non-transgenic controls. The comparative control for H01 is CE7 GS and for P01 and AS13 is CE6 GS. GA_1 is the biologically active species, which is formed from GA_{20} by 3β-hydroxylation. GA_{20} is produced from GA_{19} by GA 20-oxidase activity and is converted to the inactive GA_{29} by 2β-hydroxylation.

Young leaves

	GA_{19}	GA_{20}	GA_1	GA_{29}
CE7 GS	1.8	5.2	4.1	NF
H01	11.2*	0.2	0.9	NF
CE6 GS	NF	0.9	NF	2.8
AS13	NF	3.4	1.1	NF

Shoot tips

	GA_{19}	GA_{20}	GA_1	GA_{29}
CE7 GS	7.1	3.4	1.3	3.4
H01	14.8*	0.6	0.1	0.9
CE6 GS	NF	7.2	1.8	2.0
AS13	17.7*	NF	0.2	NF

*= concentration estimated using a linear fit of the calibration curve.

NF= not found, no quantification could be made because internal standard not recovered.

In three lines analysed, this caused mean internode length and internode number to be reduced to 60 to 70% and 80 to 90%, respectively, of those of the non-transgenic control. The overall height of the three lines ranged between 50 to 75% that of the non-transgenic control. To test whether the reduction in tree vigour would be maintained on semi-invigorating and invigorating rootstocks, transgenic dwarf scions of two lines were grafted onto MM106 and M25 stock. After one growth season, it was apparent that the dwarf scions did indeed remain dwarfed, particularly on invigorating M25 rootstock (Figures 5 and 6). Therefore, it is now possible to overcome the invigorating effects of rootstocks, thus widening the range of rootstocks available to growers around the world. Figure 6 compares the relative effects on mean overall height and mean internode length between rootstocks, expressed as a percentage of the tallest non-transgenic control means.